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How adenylate cyclase choreographs the pas de deux of the Receptors heteromerization dance

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Abstract

Our work suggests that heteromer formation, mainly involves linear motifs found in disordered regions of proteins. Local disorder imparts plasticity to linear motifs. Many molecular recognition of proteins occur between short linear segments, known as LMs. Interaction of short continuous epitopes are not constrained by sequence and have the advantage of resulting in interactions with micromolar affinities which suites transient, reversible complexes such as receptor heteromers. Electrostatic Interactions between epitopes of the GPCR involved, is the Key step in driving heteromer formation forward. The first step in heteromerization, involves phosphorylating the Ser/Thr in an epitope containing a casein kinase 1/2 (*CK1/2*)-consensus site. Our data suggests that dopaminergic neurotransmission, through cAMP dependent PKA slows down heteromerization. The negative charge, acquired by the phosphorylation of a Ser/Thr in a PKA consensus site in the Arg rich epitope, affects the activity of the receptors involved in heteromerization by causing allosteric conformational changes, due to the repulsive effect generated by the negatively charged phosphate. In addition to modulating heteromerization, it affects the stability of the heteromers' interactions and their binding affinity. So here we have an instance where phosphorylation is not just an *on/off switch*, instead by weakening the noncovalent bond, heteromerization acts like a rheostat that controls the stability of the heteromer through activation or inhibition of adenylate cyclase by the neurotransmitter Dopamine depending on which Dopamine receptor it docks at.

Keywords

Dopamine Receptors; Adenosine Receptor; Receptor heteromers; Noncovalent Interactions; Disordered Proteins; phosphorylation

Introduction

Agnati and Fuxe first pointed to the fact that G protein coupled receptors (GPCR) could interact in the cell plasma membrane resulting in receptor heteromers^{4,10}. Woods previously demonstrated that epitopes containing two or more adjacent Arg residues will form a noncovalent complex (NCX) with ones containing a phosphorylated amino acid residue through an electrostatic bond³. GPCR such as the D2R/D4R and A2AR do form heteromers through this type of Coulombic interaction²⁴⁻²⁷ between the guanidinium groups of two adjacent arginine (Arg) residues and the phosphate group of a phosphorylated Ser/Thr

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(Figure 1a and b). These findings were confirmed by Łukasiewicz¹³ (Łukasiewicz et al., 2009). Heteromers involving other receptors have also been demonstrated^{12,13}

It was argued that if heteromerization was driven by Coulombic interactions (unlike charges attract and like charges repel), it would be non-specific, as any phosphorylated Ser/Thr residue (*negatively* charged) would interact with epitopes containing multiple Arg (*positively* charged). Thus these interactions would take place indiscriminately between any proteins containing these motifs. However, we found that the first step in heteromerization, involves phosphorylating the Ser/Thr contained in a casein kinase 1/2 (*CK1/2*)-*consensus site*^{16,26,27} in the A2AR in the case of the D2R/D4R-A2AR, and in D1R in the case of the D1R-NMDAR NR1 subunit heteromers. Phosphorylation drives the interaction forward²⁵⁻²⁷, and when we used CK1/2 inhibitors the interaction failed¹⁶. Tobin demonstrated that kinases could phosphorylate and regulate GPCR and other receptors²³. We also noted that in all cases of heteromer formation, the GPCR domain containing the Arg rich epitope has downstream and less often upstream from the adjacent Arg residues, Ser/Thr that are located in a protein kinase A (*PKA*)-*consensus site*, *i.e.* they are phosphorylated by PKA^{25,26} (Figure 2).

In addition proteins or at least protein domains that get phosphorylated are intrinsically disordered^{1,3,6,7,27}, *i.e.* their amino acid composition as well as their sequence complexity, hydrophobicity, charge and other properties of the regions surrounding the residues to be phosphorylated are very similar to those of intrinsically disordered proteins. These regions contain a high percentage of disorder promoting amino acids (A, R, G, Q, S, P, E, D and K)^{3,6,7}, mainly hydrophilic residues and proline, which also promotes the localization of these sequences at the surface of the protein, thus making phosphorylation sites accessible to kinases and phosphatases. This fact provides strong support for the hypothesis that phosphorylation predominantly occurs within proteins' intrinsically disordered regions¹¹. These disordered regions also contain PEST motifs,¹⁵⁻¹⁶ rich in proline (**P**), glutamic acid (**E**), serine (**S**), and threonine (**T**). PEST sequences are associated with proteins that have a short intracellular half-life; hence, it is hypothesized that it acts as a signal peptide for protein degradation²⁰⁻²². According to Fuxreiter *et al.*, local disorder imparts plasticity to linear motifs [LM]⁵. At the structural level, Protein-Protein Interactions can follow one of two mechanisms; globular proteins with their well-defined three-dimensional conformation make high-affinity complexes with their partners. However, it was noted that many molecular recognition of proteins occur between short linear segments, known as LMs, such as in the case of the SH3 domain¹⁷⁻²⁰. Interaction through such linear motifs (LMs) gives an alternative, more versatile way for protein-protein interaction. Short continuous epitopes are not constrained by sequence and have the advantage of resulting in interactions with micromolar affinities which suit transient, reversible complexes such as receptor heteromers; thus explaining why LMs are primarily found in signaling pathways^{18,19}. In general, these short segments (referred to as epitopes in this manuscript) are characterized by local flexibility, and are found in disordered regions of the parent protein^{1,27}. This is a good description of most epitopes involved in heteromer formation.

Protein phosphorylation, a major regulatory mechanism in eukaryotic cells, is a reversible event and is influenced by proteins or peptides composition and environment^{11,14,18}. At least one-third of all eukaryotic proteins are estimated to undergo reversible phosphorylation^{11,14}. Phosphorylation modulates the activity of numerous proteins involved in signal transduction^{17,18} and regulates the binding affinity of transcription factors to their coactivators and DNA thereby altering gene expression, cell growth and differentiation^{6,7}. Phosphorylation sites frequently cluster within functionally important protein domains, as seen in the case of the Dopamine D2, D3 and D4 receptors where the phosphorylation sites are located in their 3rd intracellular loop²⁵⁻²⁷ and in the carboxy terminus of the Adenosine

A2A receptor. The phosphorylation of PEST motifs influences ubiquitin¹⁰ mediated protein degradation, which explains the short half-life of PEST rich proteins. With regard to the structural consequences of phosphorylation, both disorder to order and order to disorder transitions have been observed to follow the phosphorylation event^{16,25,27}. Conformational changes upon phosphorylation often affect protein function. For example, serine phosphorylation of the peptide corresponding to the calmodulin binding domain of human protein p4.1 influences the ability of the peptide to adopt an alpha-helical conformation and thereby impairs the calmodulin-peptide interaction¹¹.

Missale et al. showed that the second messenger Cyclic AMP (cAMP) is generated when the Dopamine D1 receptor (D1R) or the Adenosine A2A receptor (A2AR) are stimulated by their respective ligands, Dopamine and Adenosine, resulting in the activation of the enzyme adenylate cyclase, which generates cAMP¹⁵; while activation of the Dopamine D2R or D4R receptors inhibits adenylate cyclase reducing the cAMP levels². cAMP is known to activate Protein kinase A (a cAMP-dependent protein kinase), which goes on to phosphorylate proteins amongst which are many receptor proteins²⁶⁻²⁷. In the case of receptor heteromers, PKA is the kinase that phosphorylates the Ser/Thr adjacent to the Arg residues in the Arg rich epitope of the third intracellular loops of the D2, D3 and D4 Dopamine receptors.

It is our contention that in the case of the A2AR-D2R/D4R heteromerization, phosphorylation of the Ser/Thr adjacent to the Arg in the 3rd IL of the D2, D3 or D4 receptors, a PKA consensus site, adds a huge negative charge next to the positively charged arginines, which slows down the interaction by 40% or more²⁵⁻²⁷. In this particular case, instead of phosphorylation acting as an *On/Off* switch, we observed that when the Arg rich epitope is phosphorylated, it results in a *rheostat like* effect. This rheostat is driven by charge rather than resistance, resulting in the weakening of the interaction rather than the switching off of the interaction. The rheostat effect could be a fine tuning mechanism that might have a role in the changes brought up by heteromerization. We propose that the key molecule that drives the “rheostat effect” is cAMP.

Material and Methods

Epitopes

The peptides **RRRRKRVN**TKRSSR/ **RRRRKRVN**pTKRpSSR (D2R epitope), **PQTRRRRR**AKITG/ **PQpTRRRRR**AKIpTG (D4R epitope), **SFKRRRR**SSK/ **SFKRRRp**SSK (NMDA NR1 subunit epitope), **GpSSEDLK**KKEE (D1R epitope) and **SAQEp**SQGNT A2AR epitope), where **p** denotes a phosphorylated residue, were synthesized at the John Hopkins School of Medicine Peptide Synthesis Core Facility. Stock solutions were prepared in water at a concentration of 1 nmol/mL.

Sample mixtures: were prepared for mass analysis as follows: **SFKRRRR**SSK (10pmol/μL) + **GpSSEDLK**KKEE (50pmol/μL), **SFKRRRp**SSK (10pmol/μL)+ **GpSSEDLK**KKEE (50pmol/μL), **PQTRRRRR**AKITG (20pmol/μL)+ **SAQEp**SQGNT (100pmol/μL), **PQpTRRRRR**AKIpTG (20pmol/μL)+ **SAQEp**SQGNT (100pmol/μL), **RRRRKRVN**TKRSSR (20pmol/μL)+ **SAQEp**SQGNT (100pmol/μL), **RRRRKRVN**pTKRpSSR, (20pmol/μL)+ **SAQEp**SQGNT (100pmol/μL) in 50% ethanol.

Instrument

An Orbitrap-Velos mass spectrometer (Thermo Fisher, San Jose, CA) with a static nanospray source was used in positive ion mode for mass analysis. Spray voltages between 1.1-1.5 kV were employed and 4 μm nanospray tips (New Objective, Woburn, MA) were used. The resolving power of the instrument was selected at 60,000 FWHM for both MS and

MS² experiments. For MS experiments, one microscan at 100 ms was used for data acquisition. MS² experiments utilized one microscan at 500 ms and HCD collision energies between 0 – 30 V. For stability/disassociation curves, the relative ion intensities of each complex ion are plotted as a function of HCD collision energy, using the average of 25 scans/point. The ion intensities of each complex were normalized by the total ion current in each spectra.

Results

In order to probe the effect of phosphorylation on epitope- epitope interaction, the following epitope mixtures were analyzed: A2AR epitope (SAQEpSQGNT) with the nonphosphorylated/phosphorylated D2R epitope (**RRRRKRVNpTKR**SSR/**RRRRKRVNpTKRp**SSR); A2AR epitope (SAQEpSQGNT) with the nonphosphorylated/phosphorylated D4R epitope (PQ**TRRRRRRAKITG**/PQ**pTRRRRRRAKI**pTG); and D1R epitope (GpSSEDLKKEE) with nonphosphorylated/phosphorylated NMDAR-NR1 (S**FKRRRSSK**/S**FKRRRp**SSK). Initial MS analysis showed that all epitope mixtures readily produced several NCX ions (3⁺, 4⁺, 5⁺, 6⁺) (data not shown).

In order to better understand epitope- epitope interactions involving the presence or absence of phosphorylated amino acid residues, MSⁿ analysis were conducted on the NCX mass peaks formed between the epitope pairs listed above. In all cases, the major dissociation pathway for the NCX is the disruption of the *noncovalent bond* formed by the interaction of the *phosphate group* with the Arg residues guanidinium group, resulting in the generation of ions corresponding to the *intact* epitopes²⁵⁻²⁷ (figure 3a). An alternative pathway was observed for the noncovalent complexes between the A2AR and D2R/D4R epitopes in which the NCX complex is fragmented along the *covalent bond* between the oxygen from Ser and the phosphorus of the phosphate from the A2AR epitope^{8,9} (figure 3b). Similar pathways have been previously observed for NCXs formed by Arg and phosphorylated amino acid residues^{8,9,25-27}. In addition, this pathway was favored by 40% more in the nonphosphorylated epitopes compared to the phosphorylated ones^{26,27}. The absence of the observation of this pathway in the NCXs between D1R and NMDAR-NR1 epitopes suggests that the electrostatic attraction between them are weaker compared to the other epitopes in this study. Figure 3 shows product ion spectra for the NCX³⁺ between A2AR epitope and the nonphosphorylated/phosphorylated D4R epitope. We observed, that the [PQ**TRRRRRRAKITG** + HPO₃ + 2H]²⁺ peak in Fig 3a is more abundant when compared to the [PQ**pTRRRRRRAKI**pTG + HPO₃ + 2H]²⁺ peak in Fig 3b. This suggests that the electrostatic interaction is stronger in the A2AR + nonphosphorylated D4R complex compared to the A2AR+ phosphorylated D4R complex.

To prove the effect of phosphorylation we tested the stability of the noncovalent complex between the A2AR epitope and the non-phosphorylated and phosphorylated D2R/D4R epitopes, as well as between D1R epitope and the NMDAR NR1 non-phosphorylated and phosphorylated epitopes. This was done as follow: The molecular ions of the NCX were fragmented with increasing collision energies and the results for all NCX ions are summarized in Figure 4 and table 1. The relative ion intensities of each complex ion in Figure 4 are plotted as a function of collision energy. This also shows that the presence of phosphorylated residues in the D2R epitope **RRRRKRVNpTKRp**SSR (Figure 4a) or the D4R epitope PQ**pTRRRRRRAKI**pTG, (Figure 4b) greatly decreases its stability in the gas phase, suggesting a much weaker electrostatic interaction when the D2R/D4R epitope is phosphorylated, as according to Coulomb's law, like charges (in this case the addition of the negatively charged phosphate next to the positively charged Arg), weakens the attraction for the A2AR phosphate. Then in both cases, the A2AR and the D2R/D4R phosphorylated epitopes repel, giving less stable NCX than when the A2AR and the non-phosphorylated

D2R/D4R epitopes are involved in heteromer formation, as unlike charges (the positively charged Arginines and the negatively charged phosphate) form more stable complexes in the absence of phosphorylation on the D2R/D4R epitopes. In the D1R-NMDAR NR1 subunit case, the NCX GpSS~~ED~~LKKEE+ SFKRRRSSK and GpSS~~ED~~LKKEE + SFKRRRpSSK show only a slight difference, as the presence of a pair of positively charged lysine residues (KK) on the D1R epitope, already weakens the interaction with the NMDAR NR1 subunit epitope's Arg motif, further strengthening our hypothesis, that the stability of the interaction is governed by the electrostatic bond strength.

Discussion

We previously demonstrated that reversible protein phosphorylation²⁵⁻²⁷ is a major regulatory mechanism in receptor heteromerization, and that the region's environment, i.e. amino acid composition, sequence complexity, hydrophobicity, charge and other sequence attributes of regions adjacent to phosphorylation sites, are very similar to those of intrinsically disordered protein motifs^{1,27}. This provides strong support for the hypothesis that protein phosphorylation occurs predominantly within intrinsically disordered protein regions. At least one-third of all eukaryotic proteins are estimated to undergo reversible phosphorylation^{6,7,27}. Numerous proteins involved in signal transduction, use Phosphorylation to modulate their activity, it also regulates the binding affinity of transcription factors to their coactivators and DNA thereby altering gene expression, cell growth and differentiation²⁰. Phosphorylation sites frequently cluster within functionally important protein domains, i.e. the majority of phosphorylation sites of Mdm2 are located in its p53- and p14-ARF-binding regions^{19,20}. With regard to the structural consequences of phosphorylation, both disorder to order and order to disorder transitions have been observed to follow the phosphorylation event. Conformational changes upon phosphorylation often affect protein function. For example, serine phosphorylation of the peptide corresponding to the calmodulin binding domain of human protein p4.1 influences the ability of the peptide to adopt an alpha-helical conformation and thereby impairs the calmodulin-peptide interaction²⁰. The high disorder and secondary structure patterns detected in all the receptors examined, suggest the presence of molecular recognition elements (MoREs)^{5,19,20,27} that may mediate phosphorylation-regulated intra- and inter-domain interactions. Dunker and Fuxreiter proposed that proteins have such structural elements or features that mediate the binding events of disordered regions and that this element consists of a short linear region (LM) that undergoes coupled binding and folding within a longer region of disorder and called these features "molecular recognition elements" (MoREs)^{17-19,20,27}. We believe that the positively and negatively charged epitopes in many receptors are the molecular recognition elements involved in heteromerization²⁵⁻²⁷.

These functional interactions depend on heteromerization, between the D1R and a specific subunit of the NMDA receptor, the NR1 subunit. When the neurotransmitter Dopamine docks at the D1R; it activates adenylate cyclase (AC) generating the second messenger cyclic AMP (cAMP), which in turn activates the kinase PKA [AC → cAMP → PKA cascade] in dynorphin neurons, which modulates neuronal excitability and glutamatergic neurotransmission and induces PKA-mediated phosphorylation of a Ser downstream from the Arg motif in the NMDA NR1 subunit epitope²⁵⁻²⁷. On the other hand when Dopamine docks at the D2R/D4R it inhibits the [AC → cAMP → PKA cascade]. While, when the neurotransmitter Adenosine docks at the Adenosine A2AR, as in the case of Dopamine at the D1R, it also activates the AC-cAMP-PKA cascade resulting in the phosphorylation of the Ser/Thr adjacent to the D2R/D4R 3rd IL Arg-rich epitopes, which are the positively charged motifs involved in heteromer formation with the A2AR Ser₃₇₄, located in A2AR C-terminus, which becomes negatively charged when phosphorylated by Casein kinase 1 (CK1). As the electrostatic interaction between these charged epitopes is the first and

essential step that drives receptors to form heteromers, addition of a phosphate next to the Arg rich motifs decreases the positive charge of the epitope, resulting in the weakening of the electrostatic interaction that leads to heteromer formation (figure 2). This is confirmed by the data in figure 4a and b and table 1 which suggests that the phosphorylated epitope decreases heteromerization by 40-50%

In conclusion, docking of Adenosine at the A2AR decreases the interaction with D2R/D4R, while docking of Dopamine at the D2R/D4R enhances it, as it inhibits [AC → cAMP → PKA cascade]^{2,11,15} and prevents phosphorylation of the D2R/ D4R epitope (Figure 2). Thus when Dopamine is the ligand at D2R/D4R, the electrostatic interaction is unaffected, and the heteromer formed is stable, while when Adenosine is the ligand at A2AR activation of the [AC → cAMP → PKA Cascade] leads to phosphorylation of the D2R/D4R epitope and weakening of the interaction. So what we have here is a physiological event, where phosphorylation does not act as an *on/off* switch. Instead phosphorylation of the Ser/Thr adjacent to the positively charged motif, acts as a rheostat, as it decreases the interaction without disrupting the heteromers. Our data suggests that receptor heteromer formation between D2R/D4R-A2AR is influenced by their respective ligands, the neurotransmitters Dopamine and Adenosine, through activation or inhibition of the [AC → cAMP → PKA cascade]. When Adenosine docks at A2AR or Dopamine at D1R, adenylyl cyclase is activated and cAMP is generated, which in turn activates the kinase PKA, which then phosphorylates Ser/Thr adjacent to the Arg rich sequence in the positively charged epitope. When this event occurs, the Coulombic interaction between this epitope and the negatively charged epitope on the opposing receptor is weakened. However the heteromer still exists. So in this system, instead of a phosphorylation event turning a switch *on/off*, the stability of the interaction is modulated by phosphorylation of the positively charged epitope. Addition of this negative charge diminishes the positive epitope likelihood of interacting with the negatively charged epitope on its partner receptor, by weakening the stability of the noncovalent electrostatic bond between receptors. It also suggests that ligands (Dopamine and Adenosine) have a say in heteromerization and can alter the stability of heteromers, once they are formed. In other words, the phosphorylation state sets the “gain” of the system (figure 2). It determines whether a neurotransmitter molecule, mainly Dopamine or Adenosine will be effective or not in activating a receptor, if this receptor is involved in the formation of a heteromer, as these neurotransmitters, don't just act as receptors' ligands. When Adenosine docks at the A2AR it also activates adenylyl cyclase, which then generates cAMP, which activates PKA, which then phosphorylates the Ser/Thr adjacent to the Arg rich motif, which weakens the interaction. While when Dopamine docks at the D2R or D4R, it also inhibits adenylyl cyclase. Thus no additional phosphorylation occurs on the D2R or D4R, thus preventing the disruption of the Coulombic interaction. So we could say that when Dopamine is the ligand, the D2R or D4R lead the dance, and likes to keep its partner the A2AR receptor in a tight electrostatic embrace. While when Adenosine is the ligand, A2AR leads the dance. While not quitting the dance floor, by adding a phosphate, it forces its partner the D2R or D4R to keep a respectful distance. Hence when Dopamine is around D2R or D4R are in charge, while when Adenosine is, A2AR has the upper hand, thus suggesting why the pharmaceutical properties of compounds are altered when receptors form heteromers or a mosaic.

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Heteromers form through electrostatic interactions of linear motifs located in disordered regions of receptors' proteins

Activation/inhibition of adenylate cyclase by Adenosine/Dopamine controls production of cAMP which activates PKA

Phosphorylation of Ser/Thr by cAMP activated PKA in the Arginine Rich epitope controls the stability of heteromers

Dopaminergic neurotransmission, through cAMP dependent PKA slows down heteromerization

In heteromerization, phosphorylation is not just an *on/off switch*, by weakening noncovalent bonds, it acts like a rheostat

Figure 1.

a) Epitopes location within D₂R and A_{2A}. b) The Arginine positive charge, the blue blob, engulfing the negatively charged (red) phosphate.

Figure 2. Schematic of the mechanism of the Adenosine A_{2A} Dopamine D₂ receptor interaction, showing how kinases control heteromer formation, Casein kinase as an *on/off switch* and *Protein kinase A* as a way to *slow down the attraction*.

Figure 3.

Product-ion spectra of NCX^{3+} from a peptide mixture of SAQEp**TS**QNT [A2AR] and (a) PQ**T**RRRRRAKIT [D4R] and (b) PQ**p**TRRRRRRAK**p**T [D4R] with HCD at a collision energy of 21 V.

(a)

(b)

(c)

HCD	R4KRVNTKR R4KRVNpTK R4KRVNpTK				HCD	PQTR5AKIT PQTR5AKIT PQpTR5AKI PQpTR5AKI			
	R4KRVNTKR	SSRR	RpSSRR	RpSSRR		PQTR5AKIT	PQTR5AKIT	PQpTR5AKI	pTG
	SSRR (Avg)	(StdDev)	(Avg)	(StdDev)		G (Avg)	G (StdDev)	pTG (Avg)	(StdDev)
0	0.93987	0.00376	0.94113	0.00504	0	0.94869	0.00277	0.93230	0.00336
1	0.94111	0.00315	0.94271	0.00559	1	0.95860	0.00220	0.94117	0.00282
2	0.94125	0.00309	0.94273	0.00604	2	0.95920	0.00227	0.94079	0.00324
3	0.94036	0.00267	0.94162	0.00597	3	0.95933	0.00213	0.93944	0.00288
4	0.94193	0.00394	0.94174	0.00418	4	0.95827	0.00189	0.93977	0.00265
5	0.94205	0.00286	0.93611	0.00469	5	0.95869	0.00155	0.94062	0.00337
6	0.94059	0.00375	0.92529	0.00563	6	0.95823	0.00206	0.93960	0.00291
7	0.93924	0.00311	0.89751	0.00446	7	0.95806	0.00206	0.93820	0.00237
8	0.93375	0.00427	0.84671	0.00534	8	0.95734	0.00269	0.93288	0.00253
9	0.92379	0.00323	0.74372	0.00736	9	0.95629	0.00210	0.92011	0.00204
10	0.89633	0.01122	0.58184	0.00742	10	0.95527	0.00240	0.89860	0.00252
11	0.85640	0.00397	0.38723	0.00695	11	0.95455	0.00257	0.89713	0.00268
12	0.75879	0.00511	0.22452	0.00837	12	0.95238	0.00233	0.77628	0.00399
13	0.62299	0.00681	0.08644	0.00416	13	0.94672	0.00272	0.66930	0.00507
14	0.45871	0.00626	0.02509	0.00212	14	0.93886	0.00264	0.52245	0.00366
15	0.26506	0.00837	0.00846	0.00167	15	0.92326	0.00239	0.36988	0.00523
16	0.11797	0.00703	0.00622	0.00124	16	0.89700	0.00327	0.23114	0.00324
17	0.04254	0.00292	0.00257	0.00064	17	0.85812	0.00338	0.12161	0.00317
18	0.01332	0.00118	0.00185	0.00010	18	0.79429	0.00382	0.05582	0.00173
19	0.00389	0.00073	0.00142	0.00047	19	0.71163	0.00410	0.02896	0.00111
20	0.00157	0.00057	0.00076	0.00030	20	0.59272	0.00501	0.01885	0.00199
					21	0.46345	0.00466	0.01364	0.00152
					22	0.31899	0.00456	0.01003	0.00103
					23	0.18070	0.00413	0.00783	0.00047
					24	0.09063	0.00325	0.00639	0.00041
					25	0.03868	0.00189	0.00477	0.00053
					26	0.01568	0.00121		
					27	0.00582	0.00078		
					28	0.00264	0.00064		
					29	0.00158	0.00061		
					30	0.00174	0.00046		

Figure 4.

A study of the stability of the NCX of (a) RRRRKRVNTKRSSR (●) and RRRRKRVNpTKRpSSR (■) [D2R] with SAQEpSQGNT [A2AR], NCX⁵⁺ (b) PQpTRRRRRRAKIpT (●) PQTRRRRRRAKIT (■) [D4R] with SAQEpSQGNT [A2AR], NCX³⁺ (c) SFKRRRSSK (●) and SFKRRRpSSK (■) [NMDAR NR1 subunit] with GpSSEDLKKEE [D1R], NCX³⁺, when exposed to *HCD of the NCX* at varying collision energies.

Table 1

Collision Energy (V) at signal intensity 4.5 AU, at which NCX starts dissociating

Receptor	non-phos	Phos
D2R-A2AR	15	12
D4R-A2AR	21	15
NMDAR-D1R	4	4